EMPIRICAL EVALUATION OF *IN VITRO* MEDIA COMPONENTS FOR CELL GROWTH AND SHOOT REGENERATION FROM *RUBUS* EXPLANTS

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ABSTRACT

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This paper reports the development of an *in vitro* shoot regeneration protocol suitable for use in genetic manipulation of *Rubus*. *Rubus* genotypes and explant sources were screened for callus growth and shoot regeneration when grown on a medium commonly used for *Rubus* micropropagation, with three levels each of BA and IBA used in all combinations. Complex interactions were found in response to genotype, growth regulator level, and their combinations.

Fewer leaf explants grew callus compared to stem or root explants, but shoot regeneration only occurred from leaf explants. The most responsive genotype and explant source ('Canby' red raspberry leaf explants) were used in subsequent experiments for optimisation of macronutrients and growth regulators. The highest frequencies of shoot regeneration from 'Canby' leaf explants were 25% and 20%, with 2 μM BA plus 0.5 μM NAA (Medium 1) or BA and NAA both at 5 μM (Medium 2) respectively.

The salt and organic composition of both these media was the macronutrients of Quoirin *et al.* (1977), Murashige & Skoog (1962) vitamins and micronutrients with iron at double strength, 30 g/l sucrose, 7 g/l BDH agar, pH 5.8.

KEYWORDS: *Rubus* - red raspberry - boysenberry - *in vitro* - shoot regeneration - adventitious shoots.

ABBREVIATIONS: 2i-P, 2 isopentenyladenine; BA, benzyladenine; IAA, indoleacetic acid; IBA, indolebutyric acid; NAA, naphthalene acetic acid; NOA, naphthoxyacetic acid; cv, cultivar; df, degrees of freedom; LSD, least significant difference; ns, not significant; *, significance at the 5% level; **, significance at the 1% level; ***, significance at the 0.1% level; DMSO, dimethylsulfoxide.

INTRODUCTION

Plant breeding programmes are increasingly incorporating the use of a range of techniques for the integration of foreign genes into the plant genome including *Agrobacterium*-mediated transformation, electroporation, and bombardment with DNA-coated projectiles (Gasser & Fraley 1989). Cell selection and the exploitation of somaclonal variation, as well as protoplast fusion are also used alongside traditional techniques of plant breeding to effect genetic modifications (Conner & Meredith 1989). An initial requirement for the development of efficient systems involving these techniques is the establishment of tissue culture systems that allow the adventitious regeneration of plants from single cells or protoplasts. Important components of this can involve the chemical and physical environment of the tissue culture system, as well as the choice of plant genotype and explant tissue (Conner & Meredith 1989). Rapid and efficient plant regeneration are important to allow the effective selection and regeneration of many independently genetically modified cells.

Although there are numerous reports of micropropagation systems for *Rubus* (reviewed by Snir 1988) little has been published on optimisation of *in vitro* culture of this genus for other purposes. The objective of this study was to determine suitable *in vitro* culture conditions for *Rubus*, and to develop an efficient shoot regeneration system, ultimately for use in an *Agrobacterium*-mediated gene transfer

system for berryfruit cultivars grown in New Zealand. A series of experiments was conducted where single factors of genotype, explant source, media salts, and growth regulator combinations and levels were varied one at a time. The effects of these factors on callus induction and growth, and on shoot regeneration are discussed. *Rubus* genotypes amenable for genetic manipulation at the cellular and molecular level were identified, and the major medium components (salts and growth regulators) were empirically optimised for callus induction and shoot regeneration.

MATERIALS AND METHODS

Plant cultivars used were *Rubus idaeus* cvs Canby, Glen Moy, Glen Prosen, Lloyd George, Skeena and Sumner and *Rubus ursinus* var *loganobaccus* cv Riwaka's Choice. Stock plant cultures were initiated each year from plants grown from root cuttings in a greenhouse by surface sterilising nodal explants in 0.5% sodium hypochlorite for 15 min followed by three rinses in sterile de-ionised water. These stock cultures were routinely subcultured onto fresh multiplication medium at three-weekly intervals. In the first season, Anderson's medium (Anderson 1980) was used with 30 g/l sucrose, 7 g/l BDH agar (BDH Chemicals Ltd., Poole, England), pH 5.8 before autoclaving at 103 kPa for 15 min. Subsequently, the medium consisted of the macronutrients of Quoirin *et al.* (1977), MS micronutrients with iron at double strength plus MS vitamins (Murashige and Skoog 1962), with 30 g/l sucrose, 7 g/l BDH agar, pH 5.8 before autoclaving (referred to as Q-MS medium in this paper). For maintenance of stock cultures, 0.5 mg/l (2.2 μM) BA was added before autoclaving. However, in all experiments, growth regulators were dissolved in DMSO, then filter sterilised and added after autoclaving. All cultures were grown in pre-sterilised plastic Petri dishes (15 mm high x 90 mm diameter), sealed with Parafilm^R. The incubation conditions for all cultures were continuous low light levels (30 μmol/m^2/sec) from cool white fluorescent tubes, and 22°C constant temperature.

All experiments were initiated 10 days after routine transfer of stock plant cultures. During experiments, all explants were transferred to fresh media at three-weekly intervals. In each experiment, ten replicate dishes were used, placed randomly in the growth room. Results (recorded after 4, 6 or 9 weeks depending on the growth responses being measured) were analysed using Genstat 5 (1990) for analysis of variance, with LSD's calculated.

RESULTS

EXPLANT AND GENOTYPE SCREENING EXPERIMENTS

For the first genotype screening experiment, the medium reported by Anderson (1980) for raspberry micropropagation was used. Internodal stem explants 0.5 cm long were cut from *in vitro* plantlets of six genotypes of red raspberry. These were placed horizontally onto Anderson's medium with MS vitamins, containing BA at 0, 1 or 2 mg l^{-1}, and IBA at 0, 0.5 or 1 mg l^{-1} in all possible combinations. Five stem explants were placed on each dish, with ten replicates for each treatment.

Only one explant (cv. Lloyd George on the medium containing 1 mg/l BA plus 1 mg/l IBA) grew an adventitious shoot during the incubation period. The proportion of stem explants of five red raspberry genotypes growing callus after four weeks incubation are shown in Table 1. Plant genotype, 1 rather than 2 mg/l of BA, and either level of IBA rather than no IBA, all had significant ($P < 0.001$) effects on the proportion of explants growing callus. Complex interactions between main effects were also evident. Generalisations across genotypes cannot be made but the following responses are evident. Callus growth from stem explants of the most responsive genotype, 'Canby', was consistently high (74-100% of explants) over the range of growth regulator combinations used. 'Lloyd George' and 'Skeena' gave consistently intermediate to high responses (60-100%) only when IBA was present; while the highest level of BA (2 mg/l) had a detrimental effect on callus growth from 'Glen Moy' stems. 'Glen Prosen' and possibly 'Sumner' genotypes gave comparatively poor responses in this experiment. This may reflect low potential in tissue culture, or they may require higher levels or different types of growth regulators than those used here.

A secondary consideration in selecting genotypes ultimately suitable for genetic transformation was the ease of proliferating the stock cultures. Lloyd George and Canby were both very vigorous in this respect. Therefore, these two genotypes plus the prolific boysenberry genotype, Riwaka's Choice, were used in the second screening experi-

Table 1. The effect of *Rubus* genotype and growth regulators on the tissue culture response of *in vitro* stem explants. Figures are the percentage of explants growing callus after 4 weeks incubation.

BA (mg/l)	0			1			2		
IBA (mg/l)	0	0.5	1	0	0.5	1	0	0.5	1
Genotype:									
Canby	74	100	84	80	100	80	78	82	100
Lloyd George	18	84	60	26	94	100	28	70	60
Glen Moy	72	100	84	76	92	90	62	52	30
Skeena	28	70	78	40	70	100	52	100	100
Sumner	30	60	74	30	70	60	26	70	72
Glen Prosen	16	30	62	36	30	30	18	30	70
LSD (5%)	12								
Significance of contrasts[a]									
genotype main effect		***							
BA (none vs some) main effect		ns							
BA (low vs high) main effect		***							
IBA (none vs some) main effect		ns							
IBA (low vs high) main effect		***							

[a] All interactions were significant except BA * IBA whenever the contrasts were other than presence versus absence of each regulator.

ment. For each genotype, young leaves of *in vitro* plantlets were removed and cut in half transversely with each half randomly assigned to treatments. Ten leaf explants per replicate were placed with abaxial surfaces in contact with Anderson's medium containing the same growth regulator combinations as in the first screening experiment. There were ten replicates per treatment. The incidence of callus formation and the proportion of explants regenerating adventitious shoots were recorded.

During this experiment it was clearly evident that the incidence of callus initiation was much lower from leaf rather than stem explants, and far fewer explants grew shoots than grew callus. However, shoot regeneration occurred from a number of leaf explants during the four week incubation period, compared to only one stem explant regenerating shoots in the first experiment. The data recorded after four weeks incubation showed complex single factor and interaction effects for both callus incidence and shoot regeneration (Table 2). They establish that *Rubus* genotypes behave differently in response to growth regulators, with Riwaka's Choice having the highest proportion of explants growing callus and Canby showing the highest regeneration potential. No red raspberry, and only 3% of Riwaka's Choice explants regenerated shoots in this experiment whenever BA was absent, compared to higher proportions of explants regenerating shoots when BA was present at either 1 or 2 mg/l. In the presence of BA, the presence of IBA was advantageous, but there was also a very highly significant interaction of BA level with IBA and genotype.

Media optimisation experiments

The results presented in Tables 1 and 2 showed that in general, leaf explants were superior explants to internodal stem explants and 'Canby' was the most responsive genotype. Therefore, in all subsequent media optimisation experiments, 'Canby' red raspberry was used with explants being young fully expanded leaves cut in half transversely. Each half leaf was randomly assigned to treatments, with ten half leaves placed with the abaxial surface in contact with the medium in each dish. There were five replicates of each treatment with experiments repeated in two successive years. Analysis of variance established that there was no significant difference at the 5% level between results from each year. Therefore results were pooled and analysed as 10 replicates.

Table 2. The effect of *Rubus* genotype and growth regulators on callus growth and shoot regeneration from *in vitro* leaf explants. Figures are the percentage of explants responding after 4 weeks incubation.

A. Callus incidence (%):

BA (mg/l)	0			1			2		
IBA (mg/l)	0	0.5	1	0	0.5	1	0	0.5	1
Genotype:									
Canby	0	5	8	4	50	34	10	30	40
Lloyd George	0	0	12	5	17	21	1	34	8
Riwakas Choice	0	16	19	92	25	81	10	55	82
LSD (5%)	9								

Significance of contrasts[a]	
genotype main effect	***
BA (none vs some) main effect	***
BA (low vs high) main effect	***
IBA (none vs some) main effect	***
IBA (low vs high) main effect	***

[a] All interactions were significant except BA (none vs some) * IBA (low vs high)

B. Shoot regeneration (%):

BA (mg/l)	0			1[b]			2[b]		
IBA (mg/l)	0	0.5	1	0	0.5	1	0	0.5	1
Genotype:									
Canby	0	0	0	0	15	8	10	6	7
Lloyd George	0	0	0	4	1	4	1	8	2
Riwakas Choice	0	0	3	4	0	10	0	0	4
LSD (5%)	-	-	-	6					

Significance of contrasts	
genotype main effect	***
BA main effect	ns
IBA (none vs some) main effect	*
IBA (low vs high) main effect	ns
genotype x BA interaction	ns
genotype x IBA (none vs some) interaction	ns
genotype x IBA (low vs high) interaction	***
BA x IBA (none vs some) interaction	ns
BA x IBA (low vs high) interaction	ns
genotype x BA x IBA (none vs some) interaction	***
genotype x BA x IBA (low vs high) interaction	**

[b] Analysis of variance and calculation of LSD (5%) were restricted to these treatments only because of the general lack of shoot regeneration when BA was absent.

When evaluating the inorganic composition of the culture medium, three formulations commonly used for *Rubus* micropropagation (Snir 1988, J. Seelye pers. comm.) were compared for their effects on explant survival and callus induction. The growth regulators used were 0.5 mg/l (2.2 μM) BA plus 1.0 mg/l (5.3 μM) NAA, with 30 g/l sucrose, 7 g/l BDH agar, pH 5.8 before autoclaving. After 3 weeks, the percentages of explants that had grown callus and that had senesced (*ie.* more than half the surface area of the explant was bleached or brown) were recorded (Table 3). After nine-weeks, tissue mass was weighed and growth rate calculated as:

$$\frac{\text{[final weight (mg) - initial weight (mg)]}}{\text{initial weight (mg)/63 days.}}$$

The proportion of explants with at least one shoot regenerating was also recorded. The results of this comparison are given in Table 3. This established that Q-MS medium gave consistently better results than MS medium (Murashige and Skoog 1962) basal medium, 30 g/l sucrose, 7 g/l BDH agar, pH 5.8) or the medium of Anderson (1980). Fewer explants senesced and more explants grew callus on Q-MS medium. The proportion of explants regenerating was not significantly different in response to inorganic media composition, although no regeneration occurred on MS medium. Shoot regeneration on Anderson's medium was very erratic across replicates, while on Q-MS medium a consistent low proportion of explants regenerated shoots. This superior formulation for cell growth replaced Anderson's medium as the basal medium for all subsequent experiments on growth regulators, and for stock plant multiplication.

The cytokinins BA, 2iP, kinetin and zeatin (each at 2.2 μM with NAA at 5.3 μM) and then the auxins IAA, IBA, NAA and NOA (each at 5.3 μM with BA at 2.2 μM) were screened for their effect on callus induction and tissue growth rate after four weeks. No data for regeneration of shoots are presented because of the very low proportion of explants regenerating shoots. BA + NAA gave 2% regeneration in each experiment, as did zeatin + NAA. The most favourable results (*ie.* high incidence of callusing along the cut edges of explants, rather than fewer callus initiation sites) occurred using BA as the cytokinin, and NAA as the auxin (Table 4).

The optimal concentrations of BA and NAA were determined in a factorial experiment using each growth regulator at 0, 0.5, 2, 5 or 20 μM, in all possible combinations. Callus induction, tissue growth rates, and the proportion of explants growing roots or adventitious shoots were recorded after six weeks (Table 5). All explants grew callus except for some explants in the control (no growth regulators present), so these data are not presented. Best responses in growth rate, proportion of explants regenerating shoots and the number of shoots per explant predominantly occurred when BA was present at 2 or 5 μM. Root development only occurred in the absence of BA. Analyses of variance and calculation of LSDs were therefore restricted to those treatments. Generally, tissue growth rates were low in the absence of NAA and there was a drop from peak growth rates for each level of NAA when BA was at 20 μM. With BA at 2 or 5 μM, the level of BA had a very highly significant effect on growth rates; there was a linear response to NAA, and significant interactions occurred between growth regulators. There was a quadratic root development response to NAA in the absence of BA. The only significant effect of treatments on the proportion of explants regenerating shoots, and on the number of shoots per explant was the interaction between BA (at either 2 or 5 μM) and the linear response to NAA over the range of 0-5 μM. The highest frequencies of shoot regeneration from 'Canby' leaf explants were 25% and 20% respectively, when growth regulator levels were either 2 μM BA plus 0.5 μM NAA (Medium 1), or BA and NAA both at 5 μM (Medium 2). The number of shoots per regenerating explant (calculated from Table 5) for Media 1 and 2 above were 1.2 and 1.45 respectively.

DISCUSSION

In this study *Rubus* explant sources appropriate for Agrobacterium-mediated transformation systems were chosen for developing an *in vitro* plant regeneration system. Leaf and internodal stem explants were screened. Leaf explant was more responsive than stem explant in terms of regeneration of adventitious shoots. Genotypes were screened for their rapid multiplication *in vitro* providing ample material for experiments, as well as for their responsiveness in terms of incidence of callus and the regeneration of adventitious shoots. The responses of each genotype to growth regulators varied, with some genotypes responding posi-

Table 3. Inorganic medium composition screening for growth responses from *in vitro* leaf explants of Canby red raspberry. Percentages are the proportion of explants responding after the time indicated.

Medium	Senescence (%) after 3 weeks	Callus incidence (%) after 3 weeks	Growth rate (mg/mg/day) after 9 weeks	Regeneration (%) after 9 weeks
Q-MS[a]	19	62	0.55	8
Anderson (1980)	31	44	0.52	11
Murashige & Skoog (1962)	49	24	0.38	0
LSD (5%)	11	10	0.11	11
Significance of overall *F*	***	***	*	ns

[a] Q-MS = macronutrients of Quoirin *et al.*, (1977) plus Murashige & Skoog (1962) micronutrients with iron at double strength.

tively, some negatively and some with no response (Tables 1 and 2). Interactions between genotypes and levels of each growth regulator were also significant. This indicates that for *Rubus*, growth regulator levels and combinations may have to be tailored for adventitious shoot regeneration systems for each genotype of interest.

Table 4. Effect of growth regulator type on the percentage of Canby leaf explants growing callus, and on tissue growth rates after 4 weeks.

Experiment 1:

Auxins (at 5.3 μM)	Callus incidence (% of explants)	Growth rate (mg/mg/day)
IBA	96	1.56
IAA	96	1.22
NAA	84	1.13
NOA	96	1.22
LSD (5%)	10	0.32
Significance of overall *F*	*	*

Experiment 2:

Cytokinins (at 2.2 μM)	Callus incidence (% of explants)	Growth rate (mg/mg/day)
BA	88	1.47
2iP	74	0.65
kinetin	56	0.51
zeatin	82	0.84
LSD (5%)	16	0.16
Significance of overall *F*	**	***

The most commonly used salts for micropropagation of *Rubus* spp. have been those of Murashige & Skoog (1962) (MS) (reviewed by McPheeters *et al.* 1988, Snir 1988). Anderson's medium (Anderson 1980) has been compared with MS medium for *Rubus* culture, by Anderson (1980), Avitia Garcia *et al.* (1985), Desjardins & Gosselin (1987), and Pyott & Converse (1981). However, the conclusions from those comparisons differ, possibly because of the varying genotypes used by each group. Those who compared genotypes within their studies found significant genotype differences in responses, in accord with the findings reported here. Yet another salts combination has been used successfully for *Rubus* micropropagation in New Zealand, consisting of the macronutrients of Quoirin *et al.*, (1977) with MS micronutrients but using iron at double strength (Q-MS) (J. Seelye, pers. comm.). This final formulation was found to be superior for 'Canby' red raspberry, to either MS or Anderson's media (Table 3).

Growth regulators used were the cytokinins BA, 2iP, kinetin and zeatin; and the auxins IAA, IBA, NAA and NOA. These were chosen because they cover the range of cytokinins and auxins commonly used in plant tissue culture. Specific growth regulators were selected on the basis of high incidence of callus proliferation along cut edges of the explants (Table 4); shoot regeneration occurred at very low frequency in these experiments so was not used as a selection criterion. In the final optimisation experiment (Table 5), the growth regulator

Table 5. Optimisation of BA and NAA levels for callus incidence and shoot regeneration from leaf explants of Canby red raspberry (after 6 weeks incubation). There were 10 replicates with 10 explants in each for each treatment.

BA (μM)	NAA (μM)	Growth rate (mg/mg/day)	Root development (%)	Shoot regeneration (%)	Shoots/explant
0	0	0.07	0	0	0
	0.5	0.06	21[a]	0	0
	2	0.09	45[a]	0	0
	5	0.16	41[a]	0	0
	20	0.69	26[a]	0	0
0.5	0	0.11	0	0	0
	0.5	0.23	0	5	0.05
	2	0.22	0	1	0.01
	5	0.51	0	0	0
	20	1.41	0	0	0
2	0	0.17	0	7[a]	0.08[a]
	0.5	0.59[a]	0	25[a]	0.30[a]
	2	0.33[a]	0	8[a]	0.09[a]
	5	0.97[a]	0	10[a]	0.12[a]
	20	1.36[a]	0	0	0
5	0	0.16	0	6[a]	0.10[a]
	0.5	1.10[a]	0	6[a]	0.06[a]
	2	0.85[a]	0	4[a]	0.04[a]
	5	1.05[a]	0	20[a]	0.29[a]
	20	1.46[a]	0	0	0
20	0	0.17	0	0	0
	0.5	0.33	0	1	0.01
	2	0.39	0	0	0
	5	0.55	0	0	0
	20	0.42	0	0	0
LSD (5%)		0.23	18	9	0.13

Significance of contrasts	Growth rate	Root development	Shoot regeneration	Shoots/explant
BA (2 vs 5 μM)	***	-	ns	ns
NAA (linear)	***	ns	ns	ns
NAA (quadratic)	ns	**	ns	ns
BA x NAA (linear)	*	-	**	**
BA x NAA (quadratic)	*	-	ns	ns

[a] Analysis of variance and calculation of LSD (5%) restricted to these treatments only because of poor growth rates or lack of response in other treatments.

levels ranged from 0-20 μM, again covering and exceeding the commonly used range. Results were taken after 6 rather than 4 weeks in this experiment, allowing more time for the development of adventitious shoots. Regeneration of shoots again showed a complex response to growth regulator combinations. Fourteen media gave no regeneration at all, nine gave 1-10% of explants regenerating, while two media gave relatively high proportions of explants regenerating (20% and 25%).

CONCLUSIONS

Rubus genotypes respond differently to plant growth regulators under tissue culture conditions, and explants from different parts of the plants exhibit different growth responses. Young leaves of 'Canby' red raspberry cut in half transversely, gave the highest frequency of adventitious shoot regeneration in this study. Media modifications improved this frequency further. The macronutrients of Quoirin *et al.* (1977) plus MS micronutrients with iron at double strength (Murashige & Skoog 1962) gave better results than MS or Anderson (1980) inorganic formulations. All media were used with the addition of MS vitamins (or Linsmaer Skoog vitamins + adenine sulphate 80 mg/l for Anderson's), 30 g/l sucrose and 7 g/l BDH agar. A combination of BA and NAA gave a consistently high incidence of callus along cut edges of explants. The optimal levels of these growth regulators were determined to be BA at 2 μM + NAA at 0.5 μM (Medium 1), or BA and NAA both at 5 μM (Medium 2). The best rates of shoot regeneration were achieved on these media with 25% of explants regenerating on Medium 1 and 20% regenerating on Medium 2. These media yielded 1.2 and 1.45 shoots per regenerating explant respectively.

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